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A 60-Kilodalton Protein Component of the Counting Factor Complex Regulates Group Size in *Dictyostelium discoideum*

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Much remains to be understood about how a group of cells or a tissue senses and regulates its size. *Dictyostelium discoideum* cells sense and regulate the size of groups and fruiting bodies using a secreted 450-kDa complex of proteins called counting factor (CF). Low levels of CF result in large groups, and high levels of CF result in small groups. We previously found three components of CF (D. A. Brock and R. H. Gomer, Genes Dev. 13:1960–1969, 1999; D. A. Brock, R. D. Hatton, D.-V. Giurgiutiu, B. Scott, R. Ammann, and R. H. Gomer, Development 129:3657–3668, 2002; and D. A. Brock, R. D. Hatton, D.-V. Giurgiutiu, B. Scott, W. Jang, R. Ammann, and R. H. Gomer, Eukaryot. Cell 2:788–797, 2003). We describe here a fourth component, CF60. CF60 has similarity to acid phosphatases, although it has very little, if any, acid phosphatase activity. CF60 is secreted by starving cells and is lost from the 450-kDa CF when a different CF component, CF50, is absent. Although we were unable to obtain cells lacking CF60, decreasing CF60 levels by antisense resulted in large groups, and overexpressing CF60 resulted in small groups. When added to wild-type cells, conditioned starvation medium from CF60 overexpressor cells as well as recombinant CF60 caused the formation of small groups. The ability of recombinant CF60 to decrease group size did not require the presence of the CF component CF45-1 or countin but did require the presence of CF50. Recombinant CF60 does not have acid phosphatase activity, indicating that the CF60 bioactivity is not due to a phosphatase activity. Together, the data suggest that CF60 is a component of CF, and thus this secreted signal has four different protein components.

There is extensive evidence that many mammalian cells secrete autocrine factors that allow the cells to sense the number of cells in a group or tissue, and they use the factor in a negative feedback loop to regulate the size of the tissue (19, 21). Autocrine cell density-sensing or cell number-sensing factors (also called quorum-sensing factors) appear to inhibit the proliferation of metastatic foci in cancer, so identifying such factors and their associated signal transduction pathways could lead to ways to inhibit tumor growth (10, 23, 30). Due to the complexity of mammalian systems and the existence of multiple paracrine factors, and despite a great deal of effort, in most cases the autocrine factors that regulate tissue or group size have not been identified.

A simple model system that has many advantages for the study of autocrine secreted factors is the eukaryote *Dictyostelium discoideum*. *Dictyostelium discoideum* cells are normally individual motile cells that eat bacteria on soil surfaces (16, 18, 26, 28, 29, 35). In the presence of a food source, the cells grow and divide. The cells initiate development when they overgrow the bacteria and consequently starve. To get at least some of the cells to a different patch of soil, the starving cells cooperatively form multicellular structures called fruiting bodies, consisting of a mass of spores on top of a 1- to 2-mm-high column of stalk cells. The spores are dispersed by the wind, and a germinating spore can then start a new colony.

Several autocrine factors coordinate *Dictyostelium* cell development. The growing cells secrete polypeptide signals, and as the cell density increases, the concomitant increase in the concentration of the signals allows the cells to sense that they are nearing starvation and causes them to begin expressing early development genes (12–14, 27, 33, 34). When the cells overgrow the bacteria and starve, they begin secreting an 80-kDa glycoprotein called CMF (11, 15, 22, 25, 43). CMF allows cells to sense the local density of starving cells, and when there is a high density of starving cells, as indicated by a high level of CMF, the cells aggregate using relayed pulses of cyclic AMP as a chemoattractant (16, 18) to aggregate as streams flowing toward a common center.

Dictyostelium cells use an autocrine factor that senses the number of cells in a stream and causes the stream to break into groups if there are too many cells in a stream (3, 5, 36). A factor that regulates group size is counting factor (CF), a 450-kDa complex of polypeptides (5). Disruption of *countin*, *cf50*, or *cf45-1*, genes encoding components of CF, results in streams not breaking up (5, 7, 8). These streams then coalesce into one huge group that forms a huge fruiting body which then collapses, spilling the spores on the ground. Conversely, adding high levels of CF or CF components to starving cells results in the formation of abnormally small groups and fruiting bodies (3–5, 7, 8). The effect of CF on group size suggests that it is part of a negative feedback loop, with a high concentration of CF inducing stream

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breakup, possibly to prevent the formation of excessively large fruiting bodies which would then collapse.

We found that preparations of partially purified CF contain eight proteins (5). We are determining whether each protein in the preparation is part of the 450-kDa complex, and is thus a component of CF, or whether it is a contaminant. In this report, we show that a 60-kDa protein that has similarity to acid phosphatases is a component of CF.

MATERIALS AND METHODS

Protein sequencing. Isolation of partially purified CF and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done as previously described (5). Tryptic digestion and sequencing of fragments of the lower band of the doublet at 60 kDa was done at the Baylor College of Medicine core facility. The potential signal sequence and cleavage site was identified using SignalP 3.0 at www.cbs.dtu.dk/services/SignalP/ (2). Potential glycosylation sites were identified using algorithms at DictyOGlyc 1.1 (www.cbs.dtu.dk/services/DictyOGlyc) and NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc).

Cell culture, group size assays, and sieving chromatography. Cell culture was done following Brock and Gomer (5) using the wild-type Ax2 strain, cells with a disruption of the *ctnA* gene (clone HDB2B/4; referred to in this and previous work as *countin*$^-$ cells) (7), *cf45-1*$^-$ cells (clone DB45-1-5) (8), *cf50*$^-$ cells (clone HDB17-4) (7), and *aprA*$^-$ cells (clone DB60T3-8) (6). Group size assays were done following Brock et al. (3). Conditioned starvation medium (CM) was prepared and concentrated following Brock et al. (7). For size fractionation, 0.3 ml of concentrated conditioned medium was loaded on a 24-ml-bed-volume Superose 12 10/300 GL gel filtration chromatography column (Amersham, Piscataway, NJ), which was run at 0.3 ml/min in PBM (20 mM KH_2PO_4, 0.01 mM $CaCl_2$, 1 mM $MgCl_2$, pH 6.1, with KOH), collecting fractions every minute. Gel filtration standards were from Sigma.

Preparation of recombinant CF60, antibody purification, and Western and Northern blots. Recombinant CF60 was prepared following the method used to prepare recombinant CF50 (7), with the exception that CGACAGCCATATGC AAAAACCATTTTATTGCC was used as the forward primer and GGATCCT CGAGTTAAAATGTCATTGTTGAATTTGAACATGC was used as the reverse primer for the PCR. This resulted in a cDNA fragment encoding the region from the first amino acid of the putative secreted CF60 protein to the TAA stop codon. Analytic ultracentrifugation was performed as previously described (20). Recombinant CF60 was used by Bethyl Laboratories (Montgomery, TX) to produce affinity-purified rabbit polyclonal anti-CF60 antibodies. Preparation of samples and staining of Western blots was done following Brock et al. (7) using the affinity-purified anti-CF60 antibodies at 0.4 μg/ml. Staining of Western blots with anti-CF50 and anti-countin antibodies was performed as previously described (7). To generate a probe for *cf60*, PCR was done on wild-type vegetative cDNA with the primers CGCGGCAGCCATATGCAAAAACCATTTTAT TGCC and GGATCCTCGAGTTAAAATGTCATTGTTGAATTTGAACA TGC. This generated 1.35 kb of full-length *cf60* cDNA. Isolation of RNA and Northern blots were performed following Brock et al. (7).

cDNA isolation and generation of a knockout construct. To generate a homologous recombination knockout construct, PCR was performed using Ax2 genomic DNA as a template. All DNA fragments were ligated into pCR 2.1 (Invitrogen, San Diego, CA) and sequenced at Lonestar Labs (Houston, TX). PCR with the primers CGATAATCATCCGCGGACCTCTTCAGGGTT AAGG and GCCGGCTCTAGAGGTGTTCTATCACCATGTCTTG yielded a 760-bp fragment of the 5′ side of *cf60*. This was digested with SacII and XbaI and ligated into the same sites in pBluescript SK+ (Stratagene, La Jolla, CA), which had been previously modified to contain the 1.4-kb XbaI-HindIII blasticidin resistance cassette from pUCBsrΔBam (1, 40) to generate pcf60-L. PCR was then done with GCCAGTGTAAGCTTTCCCATTACCAGAGGGTGTC and CGCACTAGGGCCCCACAGTGGAATTTTATTTTAATGG to generate a 992-bp fragment of the 3′ side of *cf60*. The fragment was digested with HindIII and ApaI and ligated into the same sites in pcf60-L to generate pcf60-LR. This was digested with SacII and ApaI, and the insert was purified by gel electrophoresis and a Geneclean II kit (Qbiogene, Inc., Carlsbad, CA). *Dictyostelium discoideum* Ax2 cells were transformed with the construct following the procedures of Shaulsky et al. (37). PCR was used to verify the disruption of *cf60*, but a stable/viable knockout could not be obtained.

Antisense repression and overexpression of CF60. To create a *cf60* antisense construct, a PCR was done using a vegetative cDNA library and the primers GAATGTCGACCAAAAACCATTTTATTGCCAAGC and GCATGGATCC AAATGTCATTGTTGAATTTGAAC to generate a fragment of the *cf60* coding region corresponding to the entire polypeptide, starting with the first putative amino acid of the mature protein containing an SalI site on the 5′ side and a BamHI site on the 3′ side. The cDNA generated was cloned in an antisense direction using the corresponding enzyme sites in the PV18neo vector (38, 39). Ax2 cells were transformed following the procedures of Manstein et al. (31), and expression of CF60 was verified by staining Western blots of whole-cell lysates and CMs with anti-CF60 antibodies. The resulting strain was designated DB60BAS, and in this report it is referred to as *cf60as*.

To make an overexpression construct, a PCR was done as described above using the primers GCGCTGGTACCATGATTAAAAAAAGTGCATTAA TAAC and CCGGATCTAGATTAAAATGTCATTGTTGAATTTGAAC to generate a fragment of the *cf60* coding region corresponding to the entire polypeptide, starting with the first methionine with a KpnI site on one side and an XbaI site on the other. A TAA stop codon was incorporated at the end of the coding region. After digestion with KpnI and XbaI, the PCR product was ligated into the corresponding sites of pDXA-3D (17) to produce the overexpression construct. Transformation and expression level verification was done as described above. The resulting strain was designated DB60BOE-5, and in this report it is referred to as *cf60*^{OE}. Quantitation of CF60 in CM was done using densitometry of Coomassie-stained gels, comparing band intensities to the band intensities of known quantities of bovine serum albumin (Sigma). For both Coomassie-stained gels and Western blots, 15 μl of unconcentrated 20-h CM was loaded on gels.

Phosphatase assay. To assay for phosphatase activity, 800 μl of PBM, recombinant CF60 in PBM, or P-6409 bovine prostatic acid phosphatase (PAP; Sigma) in PBM was added to 200 μl of N-7653 *p*-nitrophenyl phosphatase liquid substrate (Sigma). The reaction was incubated at 22 or 37°C for 1 h, and the reaction was stopped by adding 200 μl of 2 M NaOH. The absorbance was then read at 410 nm. Conditioned media were similarly assayed, but at 22°C only. The activity of different amounts of bovine prostatic acid phosphatase was used to make a standard curve, defining 1 U/ml of activity as the activity present in a 1-μg/ml solution of bovine prostatic acid phosphatase.

Nucleotide sequence accession number. The sequence of the *cf60* gene was submitted to GenBank under accession number DQ143912.

RESULTS

CF60 has similarity to acid phosphatases and is part of the CF complex. SDS-polyacrylamide gels of partially purified CF show a doublet at 60 kDa (5). We identified the upper band in the doublet as AprA, a component of a secreted factor that regulates proliferation of growing cells (6). AprA, however, is not a component of CF and thus was a contaminant in the CF preparation. The N terminus of the lower band of the doublet at 60 kDa was blocked (5). We obtained the amino acid sequence LGMSTFLEEVVDNIR from a tryptic peptide from this band. A search of genomic sequences showed that this is encoded by a *Dictyostelium* gene containing two introns. We named this gene *cf60*. The predicted CF60 polypeptide backbone has a molecular mass of 46.8 kDa and a pI of 4.6. The amino acid sequence starts with an apparent signal sequence, with a predicted cleavage site between amino acids 22 and 23 (Fig. 1). The predicted molecular mass and pI of the secreted protein (this starts with the sequence QKPFY) are 44.4 kDa and 4.5, respectively. A search of the National Centre for Biotechnology Information's website indicated that CF60 has a 92.7% alignment over 357 amino acids with the pfam 00382 histidine acid phosphatase motif. The secreted form of CF60 has 24% identity and 44% similarity over 292 amino acids to the *Legionella pneumophila* major acid phosphatase and 26% identity and 45% similarity over 323 amino acids to the human prostatic acid phosphatase, and it also has similarity to several acid phosphatases from other systems. However, as described below, we were unable to detect any significant amount of acid phosphatase activity associated with CF60.

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  1  MIKKSALITL FLVSLILGVS LSQKPFYCQA PEPTPSLNTD GLTLKMVQIL   50
 51  TRHGDRTPLY STLKPTMNTW DCNLGWLMVS SLNNVPGAAT DVDRLFRKVY  100
101  MPNREYFPGN CSDGQLTSLG FQQHLQLGQS LRQLYVDKYE LLPSELSVDA  150
151  ASTIWVRSTD VPRTIQSVQG HLTALFPPTT VTSGSGIPII NINTMDNYYE  200
201  NMTPNPTLCP ELAVLIANTT TTPEWGEFIT NTTQLKEDVM ETLGISVFPG  250
251  WSSLMDLFFA TQCHDFPLPE GVTQDMVTQV YEAAYWQYQY QLSFPMIARL  300
301  GMSTFLEEVV DNIRAFVNGT SSVKYIVFSG HDDSVGPFTN LFGLMKEWPP  350
351  YASHVELELW SDEKDNYFLQ FKFNGQSYTL NGCEDVMCPI DSFFETAYSI  400
401  LVPNYADACS NSTMTF*
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FIG. 1. Predicted amino acid sequence of CF60. The original tryptic peptide sequence is highlighted in black. A signal peptide sequence identified with SignalP is underlined. Potential *O*-glycosylation sites identified by DictyOGlyc are highlighted in gray, and a potential *N*-glycosylation site identified by NetNGlyc is double underlined. This sequence is available as GenBank accession number DQ143912.

To determine whether CF60 is part of CF, we used sieving gel chromatography to fractionate conditioned starvation medium (CM) from wild-type and *cf50*$^-$ cells and stained Western blots of the fractions with anti-CF60 antibodies. We previously saw that the CF components countin, CF50, and CF45 are part of a ~450-kDa complex. In the conditioned medium from *cf50*$^-$ cells, countin and CF45 are part of an ~400-kDa complex, indicating that countin, CF50, and CF45 are part of the same complex (5, 7, 8). The anti-CF60 antibodies stained a 60-kDa protein that fractionated as a broad peak at ~450 to ~200 kDa from wild-type CM and a broad peak at ~175 to ~150 kDa from *cf50*$^-$ CM (Fig. 2). The data thus indicate that CF60 is part of an extracellular 450-kDa complex whose apparent size decreases when CF50 is absent. This in turn suggests that CF60 is a component of CF.

The CF components countin, CF50, and CF45-1 are expressed in vegetative cells and during early development, when CF is secreted by cells and regulates group size, and lower levels of the mRNAs are present during later development (5, 7, 8). A Northern blot of RNA from vegetative and developing cells indicated that *cf60* mRNA is also present in vegetative cells and during the first 5 h of development, with lower levels present up to and including 25 h, when fruiting bodies have formed (Fig. 3). This indicates that CF60 is expressed when the other components of CF are expressed.

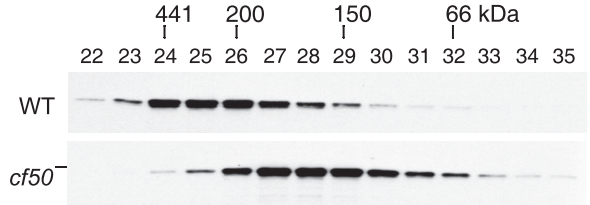

FIG. 2. CF60 is part of the CF complex. Conditioned starvation medium from wild-type (WT) and *cf50*$^-$ cells was fractionated by sieving gel filtration chromatography. Western blots of the fractions were stained with affinity-purified anti-CF60 antibodies; numbers above the lanes indicate the fraction number. Molecular mass standards eluted with peaks at the indicated fractions. Blue Dextran (~2×10^6 Da) eluted with a peak at fraction 16. For each cell line the stained band is at 60 kDa.

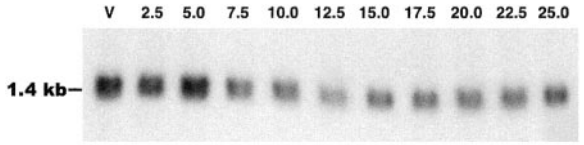

FIG. 3. *cf60* mRNA is present in vegetative and developing cells. A Northern blot of RNA from cells at the indicated times (V, vegetative cells; numbers are development time, in hours) was probed for *cf60*.

Transformants lacking CF components accumulate altered levels of the other components in cells as well as in conditioned medium (8). Compared to the levels in wild-type cells, the levels of CF60 in *cf50*$^-$ cells were slightly higher, while the levels in *countin*$^-$ cells were approximately the same as in wild-type cells (Fig. 4). Surprisingly, the CF60 levels in cells lacking AprA, a component of a different secreted factor that also affects group size, were also higher. The levels of CF60 in the conditioned medium from all three cell lines were also higher than in the wild type (Fig. 4). These data indicate that cells lacking CF components as well as cells with a disruption of other factor components have altered levels of CF60 both in the cells and in conditioned medium.

CF60 regulates group size. Despite repeated attempts, we were unable to get a stable knockout transformant. After transformation and selection for blasticidin-resistant clones in submerged liquid culture, we transferred clones to submerged liquid culture in 24-well plates. Interestingly, we did on several separate occasions obtain clones that by PCR appeared to have the genotype of a true *cf60* knockout, but these all died when there were ~2,000 cells in a well, and attempts to transfer the cells to agar plates with bacteria, submerged liquid culture, or shaking liquid culture were unsuccessful. We were able to obtain *cf60* antisense transformants (*cf60as* cells); these cells had a slight reduction in CF60 levels (Fig. 5). We also obtained cells overexpressing CF60 (*cf60*^{OE} cells); these secreted very high levels of CF60 (Fig. 5). Wild-type CM contains ~12 μg/ml total protein (22), and in the *cf60*^{OE} CM there was ~30 μg/ml

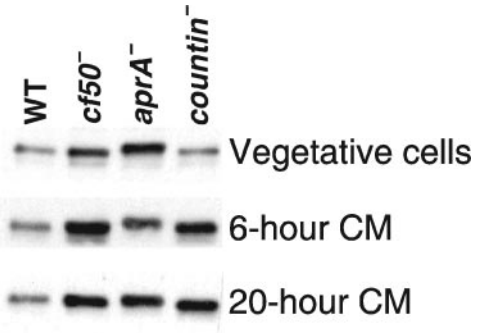

FIG. 4. Disruption of *cf50*, *aprA*, and *countin* affects the levels of CF60. Western blots of vegetative cells or conditioned starvation medium (CM) harvested at the indicated times from the indicated cell types (WT, wild type) were stained with affinity-purified anti-CF60 antibodies. The bands shown are the 60-kDa bands stained with the anti-CF60 antibodies. The presence of approximately equal amounts of protein from the different cells was verified by Coomassie staining of duplicate gels; the amount of protein in the CMs was similarly verified by silver staining duplicate gels. Data are representative of three independent experiments.

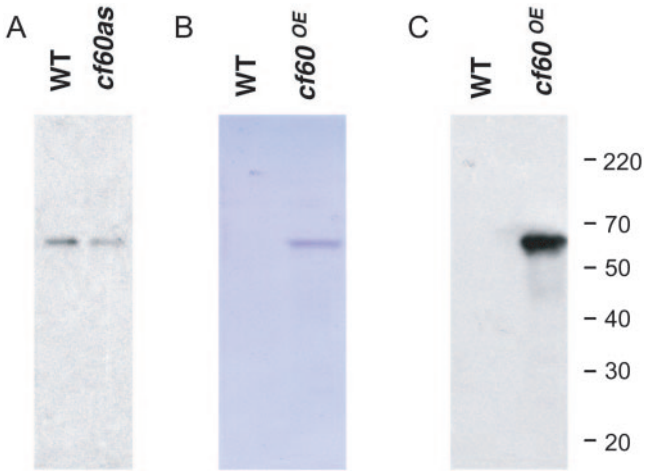

FIG. 5. Antisense repression of *cf60* decreases the amount of secreted CF60, while overexpressing CF60 has the opposite effect. A) A Western blot of CM from wild-type (WT) and *cf60as* cells was stained with affinity-purified anti-CF60 antibodies. B) An SDS-polyacrylamide gel of CM from WT and $cf60^{OE}$ cells was stained with Coomassie. Because of the low protein concentration in CM (22), no bands are visible in the WT lane. C) A Western blot of WT and $cf60^{OE}$ CM was stained as described for panel A, with the exception that a much shorter exposure time was used so that no band is visible in the WT CM. Size markers, in kilodaltons, are at the right.

of CF60. During development, the *cf60as* cells formed long elongated groups that were larger than the groups formed by wild-type cells, while the $cf60^{OE}$ cells formed large numbers of abnormally small groups, although some large groups were also observed (Fig. 6).

CF60 acts as an extracellular signal to regulate group size. To determine if the effect of overexpressing CF60 on group size is due to a secreted factor, we developed wild-type cells in the presence of conditioned starvation medium (CM) from *cf60as* cells and $cf60^{OE}$ cells. Compared to buffer alone, CM from wild-type cells had little effect on the number or size of groups formed by wild-type cells. CM from *cf60as* cells slightly decreased group number (increasing group size), and CM from $cf60^{OE}$ cells significantly increased group number (decreasing group size; Fig. 7). We have previously observed that the number of groups formed by wild-type cells can vary from month to month. The assays shown in Fig. 7 were done several months after the assays shown in Fig. 6 and several months before the assays done in Fig. 8, and thus the number and size of groups formed by wild-type cells is different in each assay.

Recombinant countin, CF45-1, and CF50 all decrease group size when added to wild-type cells (4, 8, 20). We prepared a recombinant version of the secreted portion of CF60 to simi-

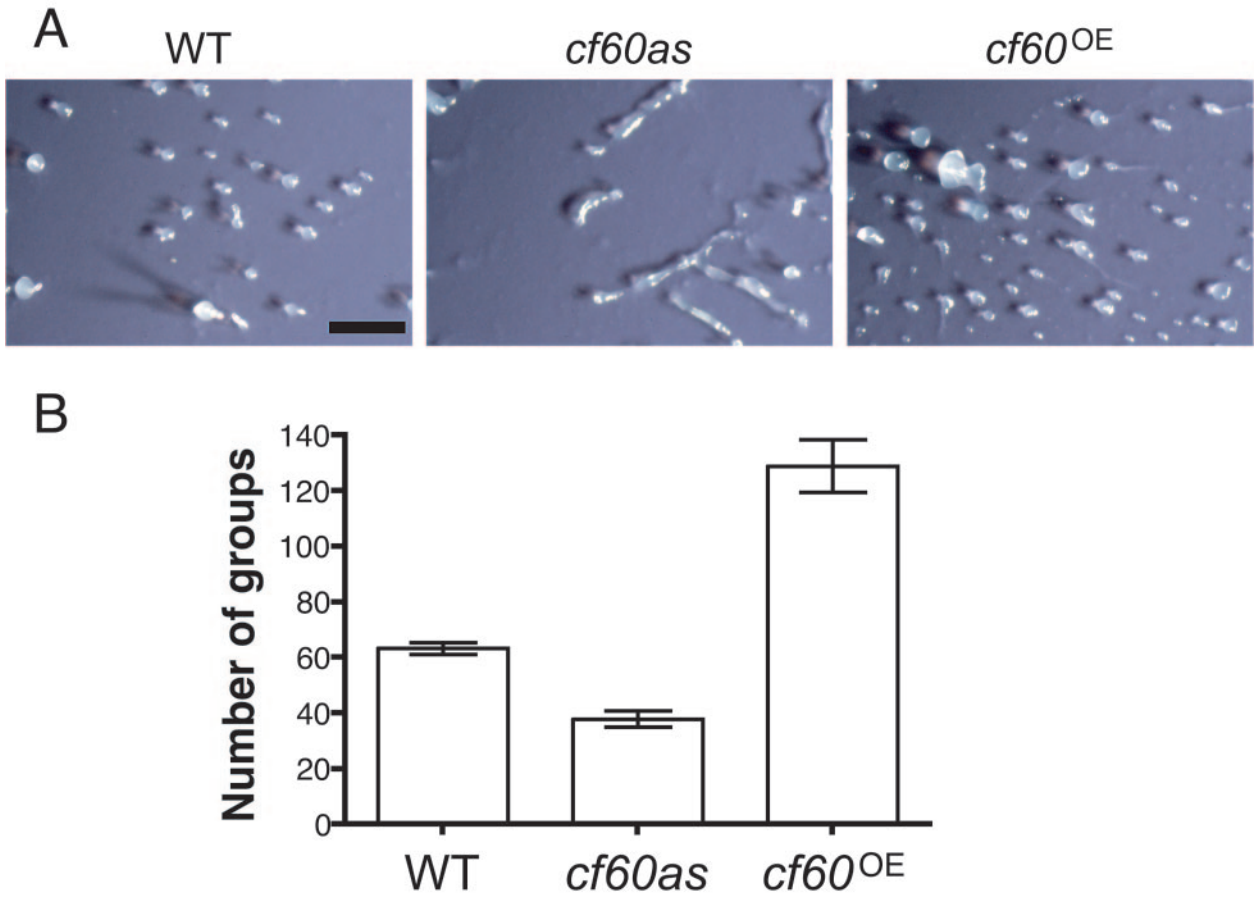

FIG. 6. Altering the levels of CF60 affects group size. A) Cells of the indicated cell type (WT, wild type) were starved on filter pads and allowed to aggregate, and photographs were taken 20 h after starvation. Bar, 0.5 mm. B) The number of groups was counted for each cell line. Values are means ± SEM ($n = 7$). The difference between WT and *cf60as* is significant, with $P < 0.05$, and the difference between WT and $cf60^{OE}$ is significant, with $P < 0.01$ (one-way analysis of variance, Dunnett's test).

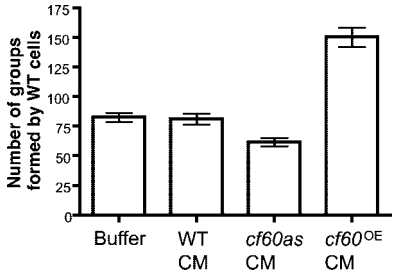

FIG. 7. Altering the levels of CF60 changes the ability of cell exudates to affect group size. Filter pads were soaked with either PBM (Buffer) or conditioned medium prepared from the indicated cell types (WT, wild type). Starved wild-type cells were then placed on the filter pads, and the numbers of groups were counted after 20 h. Values are means ± SEM ($n = 3$). The difference between WT CM and either buffer or *cf60as* CM is not significant, and the difference between WT CM and *cf60*^{OE} CM is significant, with $P < 0.01$ (one-way analysis of variance, Dunnett's test).

larly test whether it can affect group size. The recombinant protein had an apparent molecular mass of 43 kDa, close to the predicted 44-kDa mass (Fig. 8). Ultracentrifugation of recombinant CF60 indicated that it forms a dimer with an apparent mass of 83 kDa. Recombinant CF60 decreased group size and increased group number when added to developing wild-type cells (Fig. 8). Recombinant CF60 also slightly decreased group size and increased group number when added to cells lacking CF45-1 or cells lacking countin. At 1 ng/ml, recombinant CF60 increased group number by 34% when added to wild-type cells, 23% when added to *cf45-1*$^{-}$ cells, and 60% when added to *countin*$^{-}$ cells. However, when added to cells lacking CF50, there was no significant effect on group size or number (Fig. 8). Together, the data suggest that CF60 acts as an extracellular signal that negatively regulates group size, and that the effect of CF60 on group size requires the presence of CF50.

Recombinant CF60 does not have phosphatase activity. Because CF60 has sequence similarity to phosphatases, we assayed the phosphatase activity of recombinant CF60 as well as the conditioned medium from wild-type and *cf60*^{OE} cells. Using a variety of buffers and temperatures, we were unable to detect phosphatase activity associated with recombinant CF60. However, whereas wild-type CM had 5.1 ± 0.1 U/ml of phosphatase activity, *cf60*^{OE} CM had 6.7 ± 0.1 U/ml of activity (means ± standard errors of the means [SEM]; $n = 5$). The increased phosphatase activity seen in the *cf60*^{OE} CM suggests that either the CF60 secreted into the CM by the *cf60*^{OE} cells has some phosphatase activity or that overexpressing CF60 causes an increase in the secretion of some other phosphatase.

DISCUSSION

We found that CF60 is a component of CF and that CF60 decreases group size, but it requires the presence of CF50 for this effect. We were able to obtain cells with a disruption of the *cf60* gene, but these cells always quickly died. One possible explanation for this is that CF60 has a necessary function during vegetative growth and that for a few cell divisions after transformation, residual amounts of either CF60 or a metabolite regulated by CF60 were present in the transformed cells and kept them alive. Further dilution or loss of CF60 or the metabolite as the cells grew and divided might have then caused cells to die. We have previously used antisense to reduce levels of other proteins to barely detectable levels (3, 39, 42), whereas using the same vector we were only able to slightly reduce CF60 levels. Although there are many possible reasons for this, one is that CF60 is necessary for growth and proliferation.

CF is a ~450-kDa complex of at least four different polypeptides. In addition to CF60, known components of CF are countin, CF50, and CF45-1. Western blots of sieving gel chromatography

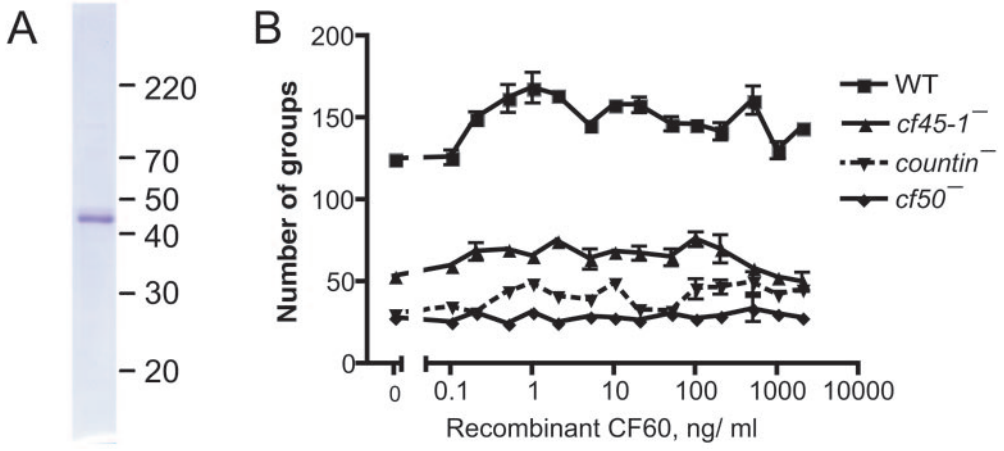

FIG. 8. Recombinant CF60 decreases group size and increases group number. A) A Coomassie-stained SDS-polyacrylamide gel of recombinant CF60 shows a single band at 43 kDa. B) Filter pads were soaked with the indicated concentrations of recombinant CF60, and the indicated cell types were starved and allowed to develop on the pads. WT, wild type. After 18 h, the number of groups was counted. Values are means ± SEM from 12 (WT), 7 (*cf45-1*$^{-}$), 3 (*countin*$^{-}$), or 5 (*cf50*$^{-}$) separate assays. The absence of error bars indicates that the error was smaller than the plot symbol. Compared to no addition, 1 ng/ml recombinant CF60 significantly increased the group number of wild-type, *cf45-1*$^{-}$, and *countin*$^{-}$ cells ($P < 0.005$; one-way analysis of variance, Dunnett's test). No amount of recombinant CF60 significantly increased group number in *cf50*$^{-}$ cells (using either *t* tests or one-way analysis of variance).

fractions of CM stained with antibodies against CF components indicate that there is very little free CF60, CF50, CF45-1, or countin in wild-type CM, indicating that in the extracellular environment of wild-type cells the CF components are in a complex (4, 8, 20). countin has similarity to amoebapores, polypeptides that form pores in target cell membranes (32, 45). countin has some amoebapore activity at low pH (20). CF50 and CF45-1 both have similarity to lysozyme, although CF50 has barely detectable lysozyme activity and CF45-1 has no detectable lysozyme activity (7, 8). Like CF45-1, CF60 appears to have evolved from an enzyme but has lost the enzymatic activity. CF thus contains proteins with similarity to amoebapores, lysozyme, and acid phosphatase, and there may be other proteins that we have not identified that are also part of CF. One possibility is that CF was originally a complex of enzymes, perhaps defensive enzymes.

The predicted and observed molecular mass of the CF60 polypeptide backbone is considerably less than the mass of the secreted protein. We have observed this discrepancy in countin, CF50, CF45-1, and a different secreted factor, CMF (4, 8, 20, 25). For the latter, we found that the protein is glycosylated, as has been found for many of the proteins that *Dictyostelium* cells secrete (41, 44). It is thus possible that CF60 is also glycosylated.

Whereas partially purified CF can more than double the group number when added to developing wild-type cells, recombinant CF60 only increased group number by ~34%, indicating that it does not have the full activity of the CF complex. The other CF components also have partial but not full CF activity (4, 8, 20). One possible explanation for this is that the individual CF components are somehow bound to cells and only a bioactive CF complex is released into CM, so that adding any component drives the equilibrium toward the release of bioactive CF. We observed that the CF components countin and CF50 have both unique and overlapping effects on cells, indicating that they activate distinct signal transduction pathways (4, 7, 20). A second possibility is that each CF component has a separate but additive effect on cells. In either case, it appears that a signal that mediates cell number counting to regulate tissue size contains at least four different proteins.

CF60 has similarity to human prostatic acid phosphatase (PAP). This protein is secreted by prostate cells into the seminal plasma and is also found in serum, with high levels in serum generally correlating with prostate cancer (24). Immunotherapy directed against PAP can in some cases cause a remission of prostate cancer (9). However, whether PAP is involved in cell number counting or group size regulation is unknown.

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